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Substantial improvements in the prevention, detection and treatment of breast cancer require the addition of new laboratory-trained investigators. The objective of this predoctoral training program is to attract new investigators into the field of breast cancer research and to provide them with an interdisciplinary predoctoral training experience focused on this malignant disorder.

The Program comprises a graduate faculty of 33, who are members of 12 departments at the University of Pittsburgh and are interested in breast cancer and graduate education. An infrastructure has been developed to allow graduate students entering any of the 7 Institutional Ph.D. granting programs to be selected for membership in this Predoctoral Training Program. The Predoctoral Training Program in Breast Cancer Biology and Therapy requires a core of 32 credits of formal course work, including participation in an ethics course, a weekly seminar/research-in-progress series and a newly constructed interdepartmental course on Breast Cancer Biology and Therapy. A minimum of 72 credits with a cumulative grade point average of at least 3.0 must be obtained prior to graduation. Student retention and progress is monitored by the Breast Cancer Training Grant Executive Committee.

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FOREWORD

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___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

9/25/97

PI - Signature Date

D. Table of Contents

E. INTRODUCTION

1. Background

Grants to support training of predoctoral students are usually given to a particular training program in an established scientific discipline or a subdiscipline, rather than for training in a specific disease entity or in a particular model system. Thus, training grants are relatively common in pharmacology, virology, immunology, epidemiology, psychology or biochemistry, regardless of the specific problems various investigators from these disciplines are addressing. What distinguishes these discipline-based predoctoral training programs from our **Training Program in Breast Cancer Biology and Therapy** is our multidisciplinary approach and the focus on a specific and important disease. The overall philosophy of this training proposal is to identify qualified graduate students in existing basic life science departments, to educate them in the problems in breast cancer and to enhance their research capabilities in this field. Our Training Program intends to expand the existing pool of investigators studying breast cancer. Moreover, the program is designed to encourage currently funded investigators to focus on breast cancer as an area of study; this is an important programmatic by-product because it fosters ongoing interdisciplinary research efforts by an array of well-funded investigators.

Breast cancer continues to be a leading cause of death of women in the United States. The magnitude of this disease demands novel approaches to improve our understanding of its etiology as well as methods of detection, prevention and treatment. Yet each year major medical and graduate schools, among them the University of Pittsburgh, continue to successfully graduate hundreds of Ph.D.s impeccably trained in basic principles of endocrinology, molecular genetics, psychology, neuroscience, biochemistry, cell biology, immunology, pharmacology and epidemiology, who have little appreciation for the contemporary problems and advances related to breast cancer. Most of these students have been narrowly trained in the details of their chosen basic science discipline. Very few of these recent Ph.D. graduates pursue postdoctoral training in breast cancer and fewer yet go on to establish successful research laboratories devoted to breast cancer. Part of the problem is the severe shortage of suitable mentors for these students, who could guide them in this direction. Our **Training Program in Breast Cancer Biology and Therapy** is designed to alter this situation. It encourages successful and enthusiastic investigators in various disciplines to address questions related to breast cancer in part by giving them students, whom they can educate while participating in the project; the program emphasizes the multidisciplinary approach to the problem; it educates many young investigators, who would have otherwise worked on other projects, on the importance of breast cancer; it emphasizes the intrinsically interesting biological questions raised by breast cancer and the potential impact and social benefit of working on breast cancer.

F. BODY OF PROPOSAL

1. Technical Objectives

a) Recruit qualified predoctoral students to the **Training Program in Breast Cancer Biology and Therapy.**
b) Educate students in the fundamental principles of breast cancer pathobiology and therapy.
c) Evaluate the progress of the enrolled students.
d) Evaluate the program and seek additional funds and resources.

2. Training Environment

The University of Pittsburgh, founded in 1787, is one of the oldest institutions of higher education in the United States. At present it comprises 16 schools having more than 2,800 faculty and 35,000 students. Of the total student population, 9,940 are currently enrolled in Ph.D. degree programs and 1752 are in professional schools of Medicine, Law or Dentistry. One hundred and fifty students currently are pursuing Ph.D. degrees at the University of Pittsburgh School of Medicine (SOM), 103 Ph.D. graduate students in the Graduate School of Public Health (GSPH) and 17 in the Biopsychology Program of the Department of Psychology in Faculty of Arts and Science (FAS).

The University of Pittsburgh Cancer Institute (UPCI) was established in 1984 to strengthen and expand cancer core and educational resources in the Western Pennsylvania region by developing new, more effective approaches to the prevention, diagnosis and treatment of cancer and by enhancing professional and lay educational programs. This is especially important because the Western PA region has the oldest population of any in the US; this extends to women. Thus, the projected incidence of tumors in the female population is extremely high. In less than 10 years the UPCI has become the major focal point for research and education not only in Western Pennsylvania but also Northern West Virginia and Eastern Ohio; it is now ranked 6th in the United States among recipients for NCI funding with more than $29 million annually; the UPCI has dedicated basic and clinical research facilities totaling more than 250,000 square feet for laboratory studies, 28,000 square feet for outpatient services and over 100 beds for cancer inpatients; over 200 women are treated annually for breast cancer at the UPCI. The UPCI has been responsible for recruiting more than 110 cancer researchers to the Institution, including both the Training Program Director and the Co-Director, and in 1990 the NCI designed the UPCI as a National Comprehensive Cancer Center. Thus, there is a cohort of young and very enthusiastic investigators available for the educational and research mission of the UPCI.

The UPCI is a Vanguard Center for the Women's Health Initiative. In addition, Carnegie Mellon University, which has faculty members in the UPCI and shares

educational programs with the University of Pittsburgh, is physically contiguous with the University of Pittsburgh. Carnegie Mellon has 7,259 students of which 1,164 are enrolled in Ph.D. programs. Thus, within a very small geographical area there is large density of students with a variety of interests and talents. This results in a very dynamic and exciting academic environment, which is conducive for interdisciplinary programs. Indeed a hallmark of the University of Pittsburgh's campus has been the successful development of joint educational programs with Carnegie Mellon University, such as the current NSF Fluorescence Center, the NSF/DoD Supercomputer Center and the Biotechnology Center. As one of the top fifteen recipients of NIH grants, the University of Pittsburgh Medical Center places particular emphasis on the importance of external research funds as a vehicle for stimulating high caliber research experience and education. Predoctoral training programs exist in all of the five basic medical science departments. The Dean of the SOM provides 22 (one year) predoctoral fellowships annually to these departments. Federal supported predoctoral funds are also available from the NIH Multi-Disciplinary Pulmonary Research Training Grant (2T32-HL07563-08; Robert M. Rogers, PI, 3 predoctoral positions), the NIH Pre- and Postdoctoral Training in Neuroscience (T32-MH18273-13; Michael J. Zigmond, PI, 4 predoctoral and 5 postdoctoral positions); Training in Neurobiology of Neurodegenerative Disease (1T32-NSO7391-01); Michael J. Zigmond, PI, 4 postdoctoral positions) and the NIH Predoctoral Training Grant in Pharmacological Sciences (GM08424-04); J. S. Lazo, PI 4 positions). Thus, there is a robust environment for interdisciplinary graduate education in and around the SOM, GSPH and FAS of the University of Pittsburgh.

3. Program Director and Participating Faculty

The **Predoctoral Training Program in Breast Cancer Biology and Therapy** was formally initiated in September 1994 with the awarding of the US Army Training Grant DAMD1-94-J-4039. The Program Director is John S. Lazo, Chair of the Department of Pharmacology and Co-Director of the UPCI Experimental Therapeutics Program, and the Co-Program Director is Olivera J. Finn, Professor of Molecular Genetics and Biochemistry and Director of the UPCI Immunology Program. Dr. Lazo has had more than 25 years of research experience in cancer biology and experimental therapeutics. Much of his early work has been directed at mechanisms of drug action and drug resistance. Most of this research has been tumor type-independent in focus. He has been a member of the Board of Directors of the American Association of Cancer Research and Chair of the 1992 Gordon Research Conference on Chemotherapy of Experimental and Clinical Cancer. He is collaborating with Dr. Olivera Finn to couple antimucin antibodies to DNA cleaving agents and is examining the role of protein phosphatases in breast cancer cells. Dr. Lazo has been a Ph.D. thesis advisor for 5 students, has been a Committee Member for 22 Ph.D. candidates and has trained 25 postdoctoral fellows; he currently is thesis advisor for 1 Ph.D. candidate, who is working on issues related to breast cancer. Two of his previous postdoctoral fellows are now investigating new anticancer agents as clinical pharmacologists at a major pharmaceutical firm (Bristol Myers Beecham) and one is

designing new diagnostic agents at a biotechnology company. Since 1976 Dr. Lazo has been intimately involved in both graduate and medical education and since 1979 he has taught a graduate level course almost every year. His basic medical science preparatory book for second year medical students published by Williams and Wilkins (*Review of USMLE Step One*) is among the most popular books of its kind and has entered its fourth edition. Writing and reviewing this book has given the Program Director a broad background in both basic and clinical issues related to malignancies including those associated with the breast. He is also PI of an NIH Predoctoral Training Grant in Pharmacological Sciences. The Co-Director, Dr. Olivera Finn, has been investigating breast cancer biology and immunology since 1985. She has trained nine graduate students and five postdoctoral fellows. Five of the students pursued research directly in the field of breast cancer and three of the postdoctoral fellows are continuing research in breast cancer in their independent positions. Dr. Finn currently has four graduate students and three postdoctoral fellows, all doing research in breast cancer. One of her graduate students is supported by an individual predoctoral fellowship in breast cancer research from the DOD and another is on our DOD training grant. One of her postdoctoral fellows is on the Susan G. Komen Fellowship for research in breast cancer. Dr. Finn participated in the Progress Review Group for Breast Cancer Meeting in Baltimore, MD, September 12-14, 1997, organized by the NIH to set research grants and priorities for the next ten years. She has also been a thesis committee member for 30 Ph.D. candidates.

The participating faculty members have been drawn from the over 175 members of the Graduate School at the University of Pittsburgh, who are eligible to train students enrolled in a Ph.D. degree granting program. We have selected these 33 faculty members by carefully evaluating them for excellence in the following categories: extramural research support; previous educational experience; research interest in cancer, particularly breast cancer; diversity of research interest and, suitability as a mentor. We have made a special effort to include a significant number of clinically trained investigators (30% of the total faculty have M.D. degrees) to ensure the appropriate exposure of students to clinically relevant issues associated with breast cancer. Participation in this training grant is not viewed as exclusionary and the Training Program Executive Committee throughout the training program funding period will consider new members. Listed below (Table 1) are the members of the faculty, their departmental affiliation and a brief description of their research interests as relate to breast cancer to illustrate the diversity of the faculty members and their interactions.

Table 1. Faculty of the Training Program

Faculty	Department	Major Research Interest
John S. Lazo, Ph.D., Program Director	Pharmacology	Chemotherapy, Drug resistance, Apoptosis
Olivera J. Finn, Ph.D., Program Co-Director	Molec. Genetics & Biochemistry/Surgery	Tumor Immunology, Mucins, Immunogenetics
Edward D. Ball, M.D.	Medicine	Bone marrow transplant., Cell surface markers
Andrew Baum, Ph.D.	Psychiatry/Psychology/Beh. Neuroscience	Behavioral medicine, Stress
Robert A. Branch, M.D.	Medicine	Clinical pharmacology, Drug metabolism
Anthony R. Caggiula, Ph.D.	Psychology	Behavioral immunology, Hormones
Andrea Cortese-Hassett, Ph.D.	Pathology	Molecular genetics, Immunology
Billy W. Day, Ph.D.	Environ. & Occupat. Health/Pharm. Sci.	Molec. toxicol., Estrogen, Computational chem.
Albert D. Donnenberg, Ph.D.	Medicine	Bone marrow transplant.
Maryann A. Donovan-Peluso, Ph.D.	Pathology	Molec. genetics, Transcription
Qing-Ping Dou, Ph.D.	Pharmacology	Cell cycle control, Cyclins, Transcription
Roy A. Frye, M.D., Ph.D.	Pathology	Oncogenes, Growth factors, Molec. biology
Joseph C. Glorioso, Ph.D.	Molecular Genetics and Biochemistry	Gene therapy
Ronald H. Goldfarb, Ph.D.	Pathology/Neurosurgery	Metastasis, Invasion, Proteases
Leaf Huang, Ph.D.	Pharmacology/Molec. Genetics & Biochem.	Liposomes, Gene therapy
Candace S. Johnson, Ph.D.	Otolaryngology/ Pharmacology	Exp. therapeutics, Cytokines, Vasculature
Daniel E. Johnson, Ph.D.	Medicine	Apoptosis
Lewis H. Kuller, M.D., Dr.P.H.	Epidemiology	Hormone metabolism, Diet, Endocrinology
Joseph Locker, M.D.	Pathology	Molecular diagnosis, Oncogenes
Michael Lotze, M.D.	Surgery/Molec. Genetics & Biochem.	Gene therapy, Immunotherapy
Susan A. McCarthy, Ph.D.	Surgery/Molec. Genetics & Biochem.	Immunology, T cell function, Apoptosis
Kenneth McCarty, M.D., Ph.D.	Pathology	Steroid receptors, Immunohistochemistry
Edward V. Prochownik, Ph.D., M.D.	Pediatrics/Molec. Genetics & Biochem.	Oncogenes, Early response genes
Paul D. Robbins, Ph.D.	Molecular Genetics and Biochemistry	Tumor suppressor genes, Gene therapy
Guillermo G. Romero	Pharmacology	Signal transduction
Herbert Rosenkranz, Ph.D.	Environmental and Occupational Health	Computational toxin analyses
Russell D. Salter, Ph.D.	Pathology	Immunology, Cell cycle proteasomes
Martin C. Schmidt, Ph.D.	Molecular Genetics and Biochemistry	Transcription factors
Said M. Sebti, Ph.D.	Pharmacology	Signal transduction, Exp. therapeutics, Drug resistance
Jill M. Siegfried, Ph.D.	Pharmacology	Growth factors, Her2/neu oncogene, Tumor vaccines
Victor Vogel, M.D.	Medicine	Biomarkers and Treatment
Theresa Whiteside, Ph.D.	Pathology/Otolaryngology	Immunology, Natural killer cells
Timothy M. Wright, M.D.	Medicine/Molec. Genetics & Biochemistry	Transcriptional regulation, Interferons
Jack C. Yalowich, Ph.D.	Pharmacology	Topoisomerases, Drug resistance, Exp. therapeutics

4. Program

The study of breast cancer biology is a complex area of investigation and further understanding of this problem as well as possible solutions will emerge only through an influx and combined effort of new investigators from many different disciplines of modern biology and science. The overall objective of our **Training Program in Breast Cancer Biology and Therapy** at the University of Pittsburgh is to exploit the well-recognized expertise of selected faculty in Endocrinology, Pharmacology, Psychology, Behavioral Medicine, Medicine, Molecular Genetics, Immunology, Cell Biology and Epidemiology and their specific interests in breast cancer. Recruitment of these investigators from their parent departments into this training program is designed to support their interests in breast cancer and to provide them with an opportunity to recruit and to train young investigators in the basic principles of their discipline using breast cancer as a specific model system.

4.1 Predoctoral Training Pool

There are nine Ph.D. granting programs at the University of Pittsburgh, School of Medicine: Bioengineering (Joint Program between the Schools of Engineering and Medicine), Biochemistry (Granted by the Department of Molecular Genetics and Biochemistry), Microbiology (Granted by the Department of Molecular Genetics and Biochemistry), Pharmacology, Pathology, Cell Biology and Physiology, and Neurobiology. Faculty of the **Training Program in Breast Cancer Biology and Therapy** are members of five of these programs. Additional faculty are from Ph.D. granting programs in GSPH, Environmental and Occupational Health and FAS, Biopsychology. Admission to the training grant program requires a bachelor's degree with a major in chemistry, biology, physics, psychology, microbiology, biology or molecular biology from an accredited college or University, with a minimum grade point average (GPA) of 3.0.

4.2 Program Administrative Structure

The administrative structure of the Training Program uses the resources of existing programs and is chaired by John S. Lazo and co-chaired by Olivera J. Finn. The routine duties such as corresponding with potential applicants, monitoring student progress, ensuring appropriate student records, distribution of information to faculty and students, seminar announcements and journal club schedules are done by the Administrator of the Graduate Program, Department of Pharmacology. This individual meets on a regular basis with the Training Program Executive Committee to evaluate student progress and program needs.

4.3 Student Recruitment and Admission

The recruitment process began in the Spring of 1994 with an effort to identify highly qualified students, who had not yet chosen their research topic or advisor (2nd year students) or who have recently identified a breast cancer related research project (3rd year students). All participating faculty received a letter informing them of the program and announcements were posted throughout the University. Particular effort was made to identify and encourage women and individuals from under represented ethnic groups to apply for these fellowship monies.

Applications for admission into the 1994 class of the **Training Program in Breast Cancer Biology and Therapy** were evaluated on June 27, 1994 by the Breast Cancer Training Grant Executive Committee. This Committee comprises the Director and the Co-Director of the program, Drs. Lazo and Finn, and five other faculty members selected for their research interests and diversities. These are: Drs. Kuller, Caggiula, Siegfried, McCarty and Whiteside. Several faculty members interviewed the candidates and provided information about the applicant, sometimes acting as a formal advocate for the applicant. Each faculty member had one vote and admission was determined based upon total votes awarded each applicant. Applicants were judged on their undergraduate record, results of GRE scores, performance in first and/or second years of graduate school, faculty comments, and a brief written statement of their research interest as it related to breast cancer. An effort was made to ensure equitable distribution of fellowships between the represented disciplines and areas of research. All awardees were notified within two weeks and no student declined the award. The Executive Committee decided to make a commitment of two years for each student pending successful completion of the first year because this would allow the student security of funding and a more meaningful graduate experience. All students were reviewed in the Spring of 1995 and the Committee renewed their support for a second year.

Applications for admission into the 1996-1997 class of the Training Program in Breast Cancer Biology and Therapy were processed as described for 1994. We received nine completed applications that were discussed in detail at the Executive Committee Meeting, August 1996 (Table 2). The six finalists are listed below (Table 3).

4.4 Course of Study

A minimum of 32 credits of formal course work and 40 credits of dissertation research are required to earn a Ph.D. in all of the participating departments. The Executive Committee has examined the progress and course grades of the students to ensure they fulfill the requirements of the Program. The students are also required to complete an Ethics Course offered by University and a special two session Ethics Conference held by Professor Lazo. The book entitled "Cantor's Dilemma" by Carl Djerassi was read by all students. Students were required to write a two-page report on issues of ethics in the life of a scientist found in the book. A discussion of student responses was

held two weeks after the reports were submitted. Dr. Vogel, a member of the Executive Committee of this Program, also convened a one day Breast Cancer Symposium (see Appendix). All students were required to attend this meeting. In the next year, a seminar series will be established to include outside speakers, who are experts in various aspects of breast cancer biology and therapy. It will also include individuals speaking on behavioral aspects of breast cancer biology and therapy.

The formal course work requirement for most of the departments is similar and is structured around the four core courses of (a) Biochemistry, Macromolecules and Metabolism, (b) Cell Structure and Function, c) Molecular Genetics and (d) Signal Transduction. The minimum course work for members of the graduate program in biopsychology are (a) Principles of Behavior, (b) Research Methods, c) Systems Neuroscience and (d) Mammalian Physiology. The second year consists of elective courses. Students supported by the **Training Program in Breast Cancer Biology and Therapy** will be required to take an additional course in breast tumor biology and therapy, which will be offered in the Spring of 1998 and taught by a member of the program faculty. This course will be entitled Breast Cancer Pathobiology and Therapy. An anticipated course outline is seen in Table 4.

Table 2

Applicants	Departments
Marni Brisson	Pharmacology
Albert R. Cunningham	Environmental and Occupational Health
Dana Dellapiazza	Pharmacology
Cheryl Fattman	Pharmacology
Robert Gealey	Environmental and Occupational Health
Amie McClellan	Biological Sciences
Jennifer Siegert	Molecular Genetics and Biochemistry
James Snyder	Molecular Genetics and Biochemistry
Kristen Veraldi	Molecular Genetics and Biochemistry

Table 3

Student	Mentor	Title of Project
Marni Brisson	Leaf Huang, Ph.D.	A cytoplasmic expression system for breast cancer gene therapy
Dana Dellapiazza	John S. Lazo, Ph.D.	Mechanisms responsible for cytoplasmic metallothionein localization in breast cancer cells
Cheryl Fattman	Qing Ping Dou, Ph.D.	Tamoxifen-induced dephosphorylation and cleavage of the retinoblastoma protein during apoptosis of human breast carcinoma cells
Amie McClellan	Jeffrey L. Brodsky, Ph.D.	The requirement for ATP and specific Hsc70 DnaJ homolog interactions for protein translocation into the yeast endoplasmic reticulum
Jennifer Siegert	Paul D. Robbins, Ph.D.	Functional interactions between the retinoblastoma protein and components of the transcription initiation complex
James Snyder	Olivera J. Finn, Ph.D.	Enchancement of T-lymphocyte effector function by constitutive expression of protein kinase C

Table 4. Course Outline for Breast Cancer, Pathobiology and Therapy

Lecture Block	Block Organizer
Breast Biology (4 sessions)	**K. McCarty**
Topics: Normal Development	K. McCarty
Abnormal Development	K. McCarty
Molecular Genetics and Markers (4 sessions)	**J. Siegfried**
Topics: Growth Factors and Signalling	J. Siegfried
Oncogenes	E. Prochownik
Suppressor Genes	J. Siegfried
Cytogenetics and Molecular Genetics	J. Locker
Invasion and Metastases (2 sessions)	**J. Locker**
Topics: Extracellular Matrix	J. Locker
Proteolytic Enzymes	J.S. Lazo
Drug Therapy/Resistance (6 sessions)	**J.S. Lazo**
Topics: Principles of Chemotherapy	J.S. Lazo
Pharmacokinetics and Chemotherapy	J. Yalowich
Cell Cycle Checkpoints	Q. Dou
Apoptosis	J.S. Lazo
Drug Resistance	J. Yalowich
Angiogenesis and Tumor Vasculature	C. Johnson
Immunobiology and Immunotherapy (6 sessions)	**O. Finn**
Topics: Tumor Antigens	O. Finn
T and B Cell Function	S. McCarthy
Natural Killer Cells	T. Whiteside
Vaccines	O. Finn
Adoptive Therapy	M. Lotze
Gene Therapy	M. Lotze
Epidemiology and Prevention (4 sessions)	**L. Kuller**
Topics: Approaches of Risk Factor Assessment	L. Kuller
Data Acquisition and Analyses	K. McCarty
Dietary Control	L. Kuller
Environmental Toxins	B. Day
Behavior (4 sessions)	**A. Caggiula**
Topics: Stress, Immunology and Hormones	A. Caggiula
Psychological Impact of Screening	A. Baum
Promoting Compliance	A. Baum
Behavior and Therapeutic Response	A. Caggiula

4.5 Student Research

A brief summary of the students' progress is given on the following pages.

A Cytoplasmic Expression System for Breast Cancer Gene Therapy

Marni Brisson
Advisor: Leaf Huang, Ph.D.

One of the most important goals in the field of gene therapy is inducing high levels of expression of transfected genes. When applying gene therapy to breast cancer, efficient expression of killer genes, cytokines, antisense molecules, etc. are essential for suppression of tumor growth. Nuclear expression by promoters such as the cytomegalovirus (CMV) promoter have been the conventional way to express transfected genes. However, it has been found that only 1% of transfected DNA reaches the nucleus where the transcription machinery resides. This leaves most of the DNA in the cytoplasm where it is eventually degraded. It would therefore be beneficial to create a system where transfected genes could be expressed in the cytoplasm, avoiding the need for nuclear delivery.

The cytoplasmic expression system was developed to express DNA in the cytoplasm using T7 RNA polymerase. This enzyme was originally delivered to cells with a reporter gene driven by the T7 bacteriophage promoter in a DC-chol:DOPE liposome formulation. This system was later improved by the creation of a T7 RNA polymerase autogene (ex. pT7 AUTO 2C-) made up of the T7 RNA polymerase gene driven by its own T7 promoter. This autogene was co-transfected to cells with a small amount of exogenous T7 RNA polymerase enzyme which would recognize the T7 promoter on the autogene to begin T7 RNA polymerase gene expression. The newly synthesized enzyme could then cycle back and recognize the T7 promoter again to continously produce T7 RNA polymerase in the cytoplasm. Though this system produced overall higher reporter gene expression, several drawbacks became apparent. Adding the initial source of exogenous T7 RNA polymerase enzyme is not only expensive but possibly immunogenic. This autogene also induced some cytotoxicity and expression levels were still not optimal.

Our goal is to create a new T7 RNA polymerase autogene which avoids the problems of previously reported autogenes while producing high levels of gene expression. This new autogene, pCMV/T7-T7pol contains a T7 RNA polymerase gene driven by both a cytoplasmic T7 and a nuclear CMV promoter. When delivered to a cell, a limited amount pCMV/T7-T7pol enters the nucleus to transcribe a small amount of T7 RNA polymerase via the CMV promoter. The T7 RNA polymerase produced then recognizes the T7 promoter in the cytoplasm to induce an autoregulatory mechanism, which continuously provides high levels of T7 RNA polymerase.

pCMV/T7-T7pol was compared with a previously used pT7 AUTO 2C- by transfections to 293 cells with a pT7-CAT reporter gene. pCMV/T7-T7pol continuously produced higher levels of reporter gene expression around 5-10 fold higher than pT7 AUTO 2C-. This expression remained at elevated levels as long as 7 days after

transfection. Expression levels with pT7 AUTO 2C- fell to baseline levels after 4 days. Western blot analysis from these cell lysates using an anti-T7 RNA polymerase antibody indicated that pCMV/T7-T7pol induced high levels of T7 RNA polymerase over the 7 day period. The T7 RNA polymerase expressed from pT7 AUTO 2C- cells were to low to be detected by these methods. pCMV/T7-T7pol induced no cytotoxic effects and expressed reporter gene activity in several different cell lines. When compared with a nuclear expression system such as pCMV-CAT, it was shown that the cytoplasmic expression system utilizing pCMV/T7-T7pol produced higher levels of CAT activity.

Though our system has shown significant improvements in gene expression over previously used cytoplasmic and nuclear expression systems, there are still many improvements that can be made. For example, the optimal transfection conditions for this system are presently being determined. We have also been focusing on the contribution of each promoter in pCMV/T7-T7 pol to the production of T7 RNA polymerase. When the T7 promoter was deleted from the autogene, there was less than a 2 fold drop in reporter gene activity. This indicated that the T7 promoter may not be functioning to its fullest potential. When observing the mRNA levels produced from each promoter by primer extension, our preliminary results indicate that the T7 promoter induces extremely high levels of mRNA exceeding that of the CMV promoter. This data indicates that the autogene expresses a substantial amount of mRNA from the T7 promoter but it is very poorly translated. This is mostly due to the fact that mRNA expressed in the cytoplasm is largely uncapped causing inefficient translation. This problem can be solved by insertion of an internal ribosome entry site (IRES) after the T7 promoter which induces cap-independent translation. Other improvements involve the addition of a Kozak sequence (a consensus sequence found in most vertebrates at the ATG start site) to initiate improved translation and a T7 terminator sequence to enhance transcription. Most of these clones have been produced and will be compared with each other and the initial pCMV/T7-T7pol autogene to find the optimal T7 RNA polymerase autogene.

Once this system is optimized, it can be used for several applications in breast cancer. One interesting prospect is the fact that pCMV/T7-T7pol already produces higher levels of mRNA in the cytoplasm from the T7 promoter as compared to the CMV promoter. Therefore, this system would be ideal for antisense and ribozyme strategies against such oncogenes as bcl2 as a possible breast cancer therapy. Our optimal goal is to eventually apply this system in vivo to use for treatments in breast cancer and other diseases.

Publications:

Li, S., Brisson, M., He, Y. and Huang, L. (1997) Gene Therapy 4:449-454.

Progress Report for Dana Dellapiazza
Advisor: John S. Lazo, Ph.D.

****

Active Processes Dictate Nuclear Localization of Metallothionein[1]

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Key Words: nucleus, metallothionein, cell compartmentation

Abstract

Nucleocytoplasmic kinesis of small proteins occurs by passive and facilitated processes that can be regulated at the level of the nuclear envelope or within the repective compartments. We examined the kinesis of metallothionein, which is a class of stress-activated proteins that function to protect cells against heavy metals, oxidants, and some electrophilic drugs. Metallothionein (6 kDa) is below the size exclusion limit for passive diffusion through the nuclear envelope, yet both nuclear and cytoplasmic metallothionein phenotypes are observed in cells. The consequences and factors controlling metallothionein subcellular partitioning are unknown. Thus, we covalently coupled metallothionein with fluorescent carboxymethylindocyanine and examined the regulation of distribution in a permeabilized cell import assay. The fluorescent metallothionein localized to the nucleus of permeabilized human SCC25 carcinoma cells, consistent with its endogenous distribution. Nuclear sequestration of the fluorescent metallothionein was inhibited by a 100-fold molar excess of unlabeled metallothionein, indicating a saturable binding mechanism. Treatment of permeabilized cells with wheat germ agglutinin inhibited nuclear localization of metallothionein, suggesting the involvement of one or more glycoproteins in this process. ATP depletion prior to permeabilization also inhibited metallothionein nuclear localization, implying an energy dependent MT nuclear translocation or nuclear retention. Chilling, however, did not inhibit nuclear sequestration

of metallothionein, supporting a passive nuclear entry mechanism. These observations imply the existence of a nuclear binding factor. Collectively, these data indicate that metallothionein passively enters the nucleus of SCC25 cells, but an active and saturable binding process to a nuclear factor is necessary for its nuclear retention.

Introduction

Proteins are partitioned in subcellular compartments by transport mechanisms that generally are active processes requiring one or more signal peptides in the transported protein as well as an organelle-specific complement of receptors. Nuclear import of karyophilic proteins is regulated by nuclear pore complexes (NPC), each pore a ~125 MDa mass structure of approximately 100 different proteins arranged in octagonal symmetry about a central gated channel (reviewed in Gorlich and Mattaj, 1996; Pante and Aebi, 1993). The central channel excludes molecules exceeding 50-60 kDa unless they display a nuclear localization sequence (NLS). Although NLSs do not share a consensus sequence, they are typified by one or more short stretches of basic amino acids and can function heterologously to direct nuclear import of attached proteins; however, the placement and number of NLSs can affect import efficiency (Kalderone et al., 1984, Dingwall et al., 1982; Dworetzky et al, 1988; Roberts et al., 1987). NLS-mediated import is saturable and therefore, receptor mediated (Goldfarb et al., 1986). It is further defined by sensitivity to ATP depletion, chilling, and agents, such as mAb414 and wheat germ agglutinin (WGA) that inactivate the N-acetylglucosamine (GlcNAc)-containing NPC proteins (i.e. nucleoporins) (Finlay et al., 1987; Davis and Blobel, 1986; Richardson et al., 1988). That alternative signaling pathways for the nuclear import exist is suggested by studies of U small nuclear ribonucleoproteins (U snRNPs), which are involved in mRNA processing. Both the 2,2,7-trimethylguanosine cap structure and binding of the common proteins to the Sm domain of U1 and U2 snRNAs are required for its nuclear translocation (Hamm et al., 1990; Fischer and Luhrmann, 1990), and import is insensitive to competition by SV40 large T antigen NLS-BSA conjugate (P(Lys)-BSA)(Michaud and Goldfarb, 1992). In contrast, nuclear import of U6 snRNPs, which do not require the g-methyl triphosphate cap structure are categorically inhibited by WGA and P(Lys)-BSA and thus, share the classical import route employed by karyophilic proteins (Michaud and Goldfarb, 1991; Fischer et al., 1991). Moreover, these import mechanisms can be distinguished by kinetic competition studies not only from each other but from nucleolar RNA-derived U3 snRNPs (Michaud and Goldfarb, 1992). Thus, it is clear that nuclear import mechanisms, or at least the import-associated receptors, differ even among classes of similar macromolecules.

Little is known regarding the nucleocytoplasmic kinesis of small molecules. Eight aqueous channels are positioned between the eight spokes that project from the outer ring subunits of the NPC to the central channel, endowing the NPC with diffusion-based transport of ions and small molecules less than ~20-40 kDa (Pante and Aebi, 1993, Peters, 1986). Possession of an NLS, however, has been shown to direct some small proteins to facilitated NPC-mediated transport rather than diffusion. Specifically, nuclear

translocation of soybean trypsin inhibitor, lysozyme and cytochrome c, all non-nuclear proteins below the size exclusion limit of the NPC, was insensitive to chilling and energy depletion; in contrast, nuclear protein histone H1 (21 kDa) import was inhibited by these manipulations, presumably due to a titratable cytoplasmic receptor (Breeuwer and Goldfarb, 1990).

Similarly, nuclear import of U1A, one of the three specific proteins associated with U1 snRNP, is sensitive to WGA and chilling and thus, exhibits the characteristics of active transport; yet its small size is more consistent with diffusion (Kambach and Mattaj, 1992). The NLS of U1A, sharing no features in common with either the SV40 large T antigen prototype (Kalderone et al., 1984) or bipartite motifs (Robbins et al., 1991) previously described, may nonetheless incur active transport. Further supporting a non-diffusional route for the nuclear import of small molecules are kinetic competition studies where P(Lys)-BSA was found to compete both with ^{35}S-labeled large and small nuclear proteins (Michaud and Goldfarb, 1993). Import inhibition was less pronounced for smaller proteins, which could not be explained by their diffusional capability, as WGA inhibited nuclear import of almost all proteins (Michaud and Goldfarb, 1993). In contrast to the ATP dependency of histone H1 and U1A import, calmodulin (16.8 kDa, CaM) enters the nucleus by a facilitated import route that is characterized as sensitive to chilling or WGA treatments but ATP dependent (Pruschy et al., 1994). Thus, the import of small proteins can be classified as diffusion-, facilitated-, or active-based. Collectively, these data suggest the availability of at least two receptor mediated nuclear import pathways for small proteins.

Metallothioneins (MT) are evolutionarily conserved, thiol-rich, stress-responsive proteins that despite their small size (6 kDa), have been observed in the nucleus or cytoplasm of many cell types and tissues (Woo et al., manuscript submitted; Banerjee et al., 1982; Nagel and Vallee, 1995). The functional significance of this nuclear localization is not known. MT present an interesting model of small protein kinesis because like CaM described above, they bind divalent metals, lack an obvious NLS, and are found in both the nucleus and cytoplasm. Previously, we investigated the regulatory mechanism governing steady state nuclear partitioning of endogenous MT in human tumor cells, and found it to be sensitive to chilling and ATP depletion (Woo et al., 1996). Here we corroborate the energy dependency of MT nuclear localization using the permeabilized cell import assay, whereby unidirectional nuclear translocation of fluorescent labeled MT was monitored in ATP depleted cells. Moreover, we demonstrate that fluorescent MT nuclear localization occurs by a saturable mechanism that is sensitive to WGA, but interestingly, is insensitive to cell chilling and anti-nucleoporin antibody, mAb414.

Materials and Methods

Cell Culture and Chemicals: HeLa (human cervical cancer), NIH3T3 (mouse fibroblast), and SCC25 (human squamous cell carcinoma) cells were purchased from American Type Culture Collection (Bethesda, MD). HeLa and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (HyClone, Logan, UT), 100 IU/ml penicillin, and 100 µg/ml streptomycin. (SCC25) were grown in supplemented DMEM and 400 µg/L hydrocortisone. All cells were grown as monolayers at 37°C in a humidified atmosphere of 5% CO_2 and routinely found to be free of mycoplasma. For these experiments, SCC25 or NIH3T3 cells were plated on glass Lab-Tek chamber slides (Nunc, Naperville, IL) one day prior to experiments. Cell culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY). Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO).

Antibody staining: Cells were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), then fixed and permeabilized for 15 minutes at room temperature in a solution of 2% paraformaldehyde, 0.1% Triton-X 100, and PBS. The cells were washed three times with ice-cold PBS and incubated for 1-2 hours at 37°C in a 10% Carnation milk solution to block for non-specific binding. Following three washes with ice-cold PBS, the cells were incubated for 2 hours at room temperature with a carboxymethylindocyanine CY3.18-conjugated, affinity purified rabbit antiserum (M2p-Cy3, approximately 5 µg/ml specific IgG concentration), which recognizes all MT isoforms and has been previously characterized (Kuo et al., 1994). Following this incubation, the cells were washed three times with ice-cold PBS, and the slide was mounted with Gelvatol and examined by fluorescence microscopy (excitation and emission; 554 nm and 568 nm). No reactivity was observed with preimmune rabbit serum or in the presence of excess MT.

Preparation of cytosolic extract: HeLa cell cytosolic extract was prepared as previously described (Yang and DeFranco, 1994). Briefly, cells were pelleted (3,000xg, 5 minutes), washed twice with ice-cold PBS, washed in ice-cold wash buffer (10 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, and 2 mM dithiothreitol), and resuspended in 1.5 volumes of ice-cold lysis buffer (5 mM HEPES, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 20 µM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each aprotinin, leupeptin, and pepstatin). After incubation for 20 minutes on ice, the cells were lysed with 10 strokes of a Dounce homogenizer. The resulting homogenate was centrifuged at 1,500xg for 15 minutes at 4°C. The supernatant was centrifuged at 4°C, 15,000xg for 20 minutes and at 100,000xg for 30 minutes. The final supernatant was dialyzed in Spectra/Por Membrane tubing (MWCO 25,000, Spectrum, Houston, TX) for 5 hours at 4°C against transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, and 1 µg/ml each aprotinin, leupeptin, and pepstatin). Extracts were frozen in a dry ice-ethanol bath, and stored at -80°C. Protein concentration of the cytosolic extracts as determined by the Bradford Assay (Biorad)

ranged from 2 to 7 mg/ml.

Preparation of fluorescent labeled MT and CaM: Carboxymethylindocyanine (Cy3) dyes are attractive fluorescent tags for small proteins, because they have a high intrinsic fluorescence intensity that is pH-independent, and a low molecular weight (FW 766). We dissolved rabbit metallothionein II (1 mg, Sigma) in 1 ml of 0.1 N Na_2CO_3 (pH 8.5) containing 0.5 mM $CdCl_2$. The solution was incubated overnight at 4°C with Cy3 monofunctional dye (absorbance max., 550 nm; emissions max, 570 nm; Biological Detection Systems, Pittsburgh, PA) to obtain a final molar stoichiometry of 2:1, Cy3:MT. The labeled protein (MT-Cy3) was purified by gel filtration using a Biogel P-4 column (Biorad, Hercules, CA) and eluted with 0.1 N Na_2CO_3 (pH 8.3). Each fraction was monitored for the presence of protein by the Bradford assay and for Cy3 by monitoring A_{552}. Based upon an extinction coefficient of 150,000 $M^{-1}cm^{-1}$ for Cy3, we calculated an average binding of one Cy3 molecule per MT in our final preparation. Synthesis of Cy3-labeled CaM (Sigma) was performed as described for MT, using a 2:1 molar stoichiometry of Cy3:CaM in the labeling reaction. MT-Cy3 (3 nmol) or unlabeled MT (30 nmol) was incubated overnight with 10,000 dpm $^{109}CdCl_2$ (Amersham, Arlington Heights, IL). Each sample was loaded on a Sephadex G-75 column (Pharmacia, Piscataway, NJ) and eluted with 0.1 N Na_2CO_3 (pH 8.5). Fractions were monitored for ^{109}Cd using a gamma counter and for Cy3 by A_{552} measurement.

Permeabilized cell import assay and cell treatments: SCC25 cells, plated as described under "Cell Culture and Chemicals", were rinsed in cold transport buffer (see "Preparation of Cytosolic Extracts"). The assay was performed essentially as described by Adam et al. (1990). Briefly, cells were permeabilized with a 5 minute incubation in 40 µg/ml digitonin at room temperature, and then rinsed with cold transport buffer. For control studies, permeabilized cells were incubated for 20 minutes with 25% cytosol, 200 pmol MT-Cy3, in ice-cold transport buffer containing 1 mM ATP, 5 mM creatine phosphate, and 20 U of creatine phosphokinase. Cells were washed with cold transport buffer, fixed with 2% paraformaldehyde, and mounted for examination by standard fluorescence microscopy. Based on previous energy depletion studies (Woo et al., 1996), the cells were preincubated in low glucose medium for 5 hours and then treated for 1 hour with 5 µM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP; Aldrich, Milwaukee, WI) and 25 mM 2-deoxy-D-glucose (2DG). The cells were treated described above for control cells, with the exception that ATP was not added to the final transport buffer. Chilling studies were performed by incubating the cells on ice for 1 hour in a humidified atmosphere of 5% CO_2 prior to permeabilization. Subsequent treatments were as described for control cells. For studies with excess MT, WGA, or mAb414, permeabilized cells were preincubated for ten minutes with a 25 mg/ml MT solution (100-fold molar excess), 5 mg/ml WGA solution, or 1 mg/ml mAb414, respectively, in transport buffer containing 1 mM ATP, 5 mM creatine phosphate, and 20 U of creatine phosphokinase. Cytosolic extract and MT-Cy3 were added, as described above.

Results

Fluorescent labeled MT binds Cd and is the same size as the native MT: An undisputed function of MT is its ability to bind Cd. Therefore, to determine if the fluorescent labeled MT remained functional, we studied the ability of the reagent to bind to $^{109}CdCl_2$ using gel filtration. As illustrated in Figure 1, covalently coupled MT-Cy3 retained its Cd binding capacity, was highly fluorescent and co-eluted from a Sephadex G-75 column with unmodified MT, consistent with the addition of only a limited number of Cy3 moieties.

MT-Cy3 localization in permeabilized cells parallels the distribution of endogenous MT: Endogenous MT localization in human SCC25 carcinoma cells and NIH3T3 mouse fibroblasts was examined using a Cy3-conjugated anti-MT antibody. Following digitonin permeabilization, the cells were fixed and incubated with the fluorescently labeled MT antibody, as outlined above. Using this method, SCC25 cells showed a nuclear MT phenotype (Figure 2A), while NIH3T3 cells showed a cytoplasmic phenotype (Figure 2C). These same two cell lines were permeabilized and incubated with MT-Cy3. The SCC25 cell line demonstrated nuclear MT-Cy3 localization (Figure 2B), while the NIH3T3 cells displayed a cytoplasmic MT-Cy3 distribution (Figure 2D). To control for the possibility that the digitonin treatment was compromising the nuclear membrane, permeabilized SCC25 cells, were incubated with a 150 kDa-FITC labeled dextran. The 150 kDa dextran (Figure 2E) and a 67 kDa-FITC BSA (data not shown) were excluded from the nucleus, indicating that the detergent treatment did not damage the nuclear membrane. Additional control studies were carried out using electroporation rather than digitonin permeabilization to study localization of these dextrans. Again, exclusion of both the 150 kDa-FITC-dextran and the 67 kDa FITC-BSA was observed, consistent with the permeabilization studies (data not shown). Finally, to control for the possibility that the localization patterns were due to intracellular compartmentation of Cy3, we incubated permeabilized SCC25 cells with unreacted Cy3. No definitive partitioning was observed (Figure 2F). Thus, the Cy3 moiety itself did not impart nuclear localization in our permeabilized SCC25 cells.

CaM-Cy3 localization is inhibited by WGA but is not affected by ATP depletion or excess MT. Like MT, CaM is a small cation binding protein that has no known NLS yet is found in significant concentrations on both sides of the nuclear envelope. Interestingly, recent studies using FITC labeled CaM have described the nuclear transport of this small protein as facilitated with an import process that is unaffected by ATP depletion, but is sensitive to treatment with WGA. Because of the superficial similarities between the two proteins, we considered the possibility that MT and CaM may share a similar nuclear import pathway. To test CaM localization in our permeabilized cell system, we labeled CaM with Cy3, as described for the fluorescent MT preparation. CaM-Cy3 localized to the nucleus of permeabilized SCC25 cells (Figure 3A). Similar to previously reported results with CaM-FITC (Pruschy et al., 1994), ATP depletion did not affect nuclear accumulation (Figure 3B), but WGA treatment did inhibit localization (Figure 3C). If MT and CaM share a similar import pathway, addition of excess MT should inhibit nuclear CaM accumulation.

Preincubation of permeabilized SCC25 cells with excess MT, however, did not affect nuclear import of CaM-Cy3 (Figure 3D). This observation indicated that these two small proteins have different nuclear localizing mechanisms.

Treatment with WGA, ATP depletors, and excess MT, but not chilling, leads to nuclear exclusion of MT-Cy3 in SCC25 cells. Because the SCC25 cell line showed nuclear MT-Cy3 localization, these cells were studied further to examine the mechanism of MT nuclear import. In contrast to the karyophilic localization of MT-Cy3 in digitonin permeabilized SCC25 cells (Figure 4A), we found FCCP and 2DG, ATP depleting reagents that block active import and binding processes, decreased MT-Cy3 nuclear localization, implying that nuclear translocation or retention was energy dependent (Figure 4B). WGA, which binds to intracellular proteins containing GlcNAc residues, inhibited nuclear localization of MT-Cy3 in SCC25 cells, suggesting a role for glycoproteins in MT nuclear concentration (Figure 4C). Treatment with 100-fold excess of MT also reduced nuclear MT-Cy3 levels, indicating a saturable process in nuclear MT-Cy3 accumulation (Figure 4D). These observations insinuated that either nuclear accumulation or retention of MT was an active, saturable process involving a glycosylated protein partner.

To explore further the mechanisms controlling MT nuclear transport or retention, we investigated the effect of the antinucleoporin antibody mAb414 on MT-Cy3 localization. As in previous experiments, SCC25 cells were digitonin permeabilized, and MT-Cy3 accumulated in the nucleus (Figure 5A). To study the effect of mAb414 on nuclear import, cells were treated with mAb414 prior to incubation with MT-Cy3. This pretreatment did not affect the sequestration of MT-Cy3 into the nucleus (Figure 5B). Since WGA interacts with more glycoproteins than mAb414, it is likely the decreased accumulation seen with WGA pretreatment was due to WGA binding to a protein other than a nucleoporin that was important in MT subcellular compartmentalization in SCC25 cells. That nuclear GlcNAc-containing proteins are abundant was confirmed by incubating permeabilized SCC25 cells with FITC-WGA. As seen in Figure 5D, WGA binding sites were seen throughout the nucleus, demonstrating the similar distribution of GlcNAc-containing proteins and MT-Cy3 in SCC25 cells. This observation supports the notion that binding to a GlcNAc-containing partner was important in nuclear distribution of MT. Finally, chilling, which blocks active and facilitated import processes, did not markedly affect nuclear concentration of MT-Cy3 (Figure 5C). This is in contrast to the nuclear import inhibition observed following dexamethasone stimulation (100 nM) of glucocorticoid receptor in SCC25 cells under identical chilled conditions (data not shown). Therefore, the failure to observe a decrease in MT-Cy3 nuclear accumulation with chilling was not due to improper experimental conditions. This suggested that import of MT-Cy3 may occur via a passive mechanism. Further evidence for passive nuclear import of MT was obtained by treating SCC25 cells with MT-Cy3 in the absence of cytosolic extract (Figure 5E). Because NPC-mediated import is reliant upon cytosolic factors (Adam et al., 1990), active transport should not occur under these conditions. Nuclear MT-Cy3 was, in fact, observed consistent with passive import of MT. Collectively, these data support a passive nuclear entry mechanism

for MT in SCC25 cells but active, saturable binding to a GlcNAc-containing partner required for nuclear retention.

Discussion

As the NPC structure continues to be elucidated, the complexities of protein kinesis become apparent. For example, how does the NPC, itself comprising roughly 500 proteins, coordinate the passage of innumerable chemically and physically distinct proteins into the nucleus? Furthermore, how does the nucleus orchestrate the transport activities of the 3000-4000 NPCs that embed a typical mammalian cell nucleus? Even less understood are questions surrounding the kinesis of small macromolecules that by virtue of their size, might be expected to distribute homogeneously throughout the cell. It is clear that gradients of ions and small molecules exist across most, if not all, cellular compartments. While membrane pumps have been identified for many ions, such mechanisms would be less practical for small proteins unless there existed substantial substrate overlap. Some small proteins, such as lysozyme, trypsin inhibitor, and cytochrome C appear to enter the nucleus by diffusion, based on their insensitivity to cell chilling or ATP depletion (Breeuwer and Goldfarb, 1990). Other small proteins, such as histone H1, enter the nucleus by a facilitated process and show a temperature sensitive, saturable, and ATP dependent import (Breeuwer and Goldfarb, 1990). The facilitated import of CaM also is sensitive to temperature and saturable, but in contrast to histone H1, refractory to ATP depletion (Pruschy et al., 1994). Our results demonstrated MT nuclear accumulation was unaffected by chilling or incubation with anti-nucleoporin antibody mAb414 indicating passive diffusion. How then are gradients maintained for MT? Studies of other proteins are informative. In *Xenopus* oocytes nuclear compartmentation of proteins does not require limiting membranes (De Robertis, 1983). Physically puncturing the nuclear envelope of oocytes alters the concentration of <5% of all 300 nuclear proteins examined (Feldherr and Ogburn, 1980). Thus, although the NPC regulates nucleocytoplasmic trafficking, it clearly is not the sole determinant of protein distribution, particularly small proteins like MT. Tethering to cell structural matrices, multimer formation or complexation with non-diffusible macromolecules may be more important in compartmentation of small molecules. Cytoplasmic retention by NLS masking or association into multimeric complexes has been demonstrated for several larger nuclear proteins including Rel-related transcription factors including NFκB; SW15, a G1-specific transcriptional regulator in *S. cerevisiae*; and the glucocorticoid receptor (Henkel et al., 1992, Moll et al., 1991, Picard and Yamamoto, 1987). Alternatively, physical attachment to the cellular matrix has been suggested for IκB, consequent to its ankyrin-like repeat motifs (Blank et al, 1992). In addition to NLS masking, the heat shock protein 90 may also localize the glucocorticoid receptor to the cytoplasm by anchoring it to cytoskeleton (Miyata and Yahora, 1991). Based on their studies of punctured nuclear membranes, Feldherr and Ogden (1980) concluded that specific nucleoplasmic binding, rather than nuclear envelope-associated barriers, is the main determinant of nuclear protein composition. Kambach and Mattaj (1992) demonstrated the cellular distribution of U1A could be altered by infusing the

nuclear or cytoplasmic compartment with excess binding sites in the form of mutant U1 snRNAs. Thus, when the U1ΔD mutant, which cannot enter the nucleus, was expressed in cells, a cytoplasmic U1A distribution was observed. Similarly, Schmidt-Zachman et al. (1993) concluded that nuclear egress of proteins is a default pathway limited by interactions with nuclear structures or the formation of large complexes.

Our data demonstrate MT nuclear localization was saturable, sensitive to ATP depletion and WGA. A broad range of WGA-binding glycoproteins exist in the nucleus (Figure 5D), and we hypothesize one or more such GlcNAc-containing proteins is a nuclear partner for MT. Although we cannot exclude the possibility of non-specific or quasifunctional (Bonner, 1975) nuclear binding, we believe the interaction was specific, as it was titratable by an excess of MT, but not by excess CaM. The possibility of suboptimal experimental conditions for cell chilling is unlikely, as we observed inhibition of dexamethasone-induced glucocorticoid receptor import into the nucleus of these cells. Furthermore, the results using mAb414 and in the absence of cytosolic extract substantiate the chilling data and indicate passive mechanisms dictate MT nuclear import.

What is the possible functional significance of nuclear MT? Leyshon-Sorland and Stang (1993), using a cryo-immunocytochemical technique, localized MT in the nuclei of liver parenchymal cells of cadmium-exposed rat to euchromatin areas. The close proximity to transcriptionally active DNA may reflect a Zn^{2+}-donating function for MT, in particular, to the substantial subset of genes whose repair or transcription relies on zinc fingers or other zinc structural motifs. In vitro transcription assays demonstrate apoMT can inactivate zinc finger transcription factors IIIA and Sp1 and further, the effect is reversible by adding exogenous zinc (Zeng et al., 1991a,b). MT may also donate Zn or Cu to other metal-dependent enzymes within the nucleus. The current studies suggest intranuclear MT-glycoprotein interactions occur, which may be important in the location-specific functionality of MT.

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Figure Legends

Figure 1: Fluorescent MT (MT-Cy3) behaves similarly to unlabeled MT, as determined by gel filtration. Rabbit MT II was incubated with Cy3 overnight at 4°C as described in "Materials and Methods". The resulting covalently modified MT (MT-Cy3) was purified with a Biorad P-4 column. Fluorescent MT (3 nmol) and rabbit MT II (30 nmol) were incubated overnight with 10,000 dpm of $^{109}CdCl_2$ and loaded on a Sephadex G-75 column. Radioactivity for the presence of ^{109}Cd and absorbance (552 nm) to detect Cy3 were determined for each fraction. V_o = void volume; bed volume of G-75 column = 90ml; □, $^{109}CdCl_2$; ◊, MT-Cy3-^{109}Cd; ○, MT-^{109}Cd; †, MT-Cy3 absorbance.

Figure 2: MT-Cy3 distribution emulates native MT. SCC25 or NIH3T3 cells were plated on 8 well chamber slides and cultured overnight. Cells were then permeabilized and incubated with a Cy3 labeled anti-MT antibody as described in "Materials and Methods". Alternatively, cells were permeabilized and incubated with fluorescent MT (MT-Cy3), a 150 kDa FITC-dextran, or Cy3 alone. *A*, anti-MT antibody, SCC25 cells; *B*, MT-Cy3, SCC25 cells; *C*, anti-MT antibody, NIH3T3 cells; *D*, MT-Cy3, NIH3T3 cells; *E*, 150 kDa FITC dextran, SCC25 cells; *F*, Cy3, SCC25 cells. Bar = 10 µm.

Figure 3: Nuclear localization of fluorescent CaM (CaM-Cy3) in permeabilized human SCC25 cells is inhibited by WGA, but not by ATP depletion or by the presence of excess MT. SCC25 cells were plated on chamber slides, cultured, and treated as described in "Materials and Methods". CaM was coupled to Cy3 (CaM-Cy3) as described in "Materials and Methods". *A*, Cells were washed, permeabilized with 40 µg/ml digitonin, and incubated with 25% HeLa cytosol, transport buffer, and CaM-Cy3; *B*, Cells were incubated in low glucose medium for 5 hours, and then treated for 1 hour with 5 µM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) and 25 mM 2-deoxyglucose (2DG). The cells were then treated as in A with the exception that ATP was not added to the final transport buffer; *C*, Cells were treated as described in A but received a 10 minute preincubation with 5 mg/ml WGA before incubation with CaM-Cy3; *D*, Cells were treated as described in panel A but received a 10 minute preincubation with 25 mg/ml MT before incubation with CaM-Cy3. Bar = 10 µm.

Figure 4: MT-Cy3 nuclear localization in permeabilized human SCC25 cells is reduced by WGA treatment, ATP depletion, and excess MT. SCC25 cells were plated on chamber slides, cultured, and treated as described in "Materials and Methods". *A*, Cells were washed, permeabilized with 40 µg/ml digitonin, and incubated with 25% HeLa cytosol, transport buffer, and MT-Cy3; *B*, Cells were incubated in low glucose medium for 5 hours, and then treated for 1 hour with 5 µM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) and 25 mM 2-deoxyglucose (2DG). The cells were then treated as in A with the exception that ATP was not added to the final transport buffer; *C*, Cells were treated as described in A but received a 10 minute preincubation with 5mg/ml WGA before incubation with MT-Cy3; *D*, Cells were treated as described in A but received a 10 minute

preincubation with 25mg/ml MT before/ incubation with MT-Cy3. Bar = 10 μm.

Figure 5: MT-Cy3 localization in permeabilized human SCC25 cells is not affected by treatment with mAb414 or chilling. Human SCC25 cells plated on glass chamber slides were washed and digitonin permeabilized as described in "Materials and Methods". *A*, Cells were incubated with 25% HeLa cytosol, transport buffer and MT-Cy3; *B,* After permeabilization, cells were treated with 1mg/ml mAb414 for 10 minutes before incubation with 25% HeLa cytosol, transport buffer, and MT-Cy3; *C,* Before permeabilization, cells were chilled on ice for 1 hour. The cells were maintained on ice throughout the procedure and were treated as described for *A*; *D,* Permeabilized cells were incubated with FITC-WGA (5mg/ml), 25% HeLa cytosol, and transport buffer. *E*, Permeabilized cells were incubated with transport buffer and MT-Cy3. Bar = 10 μm.

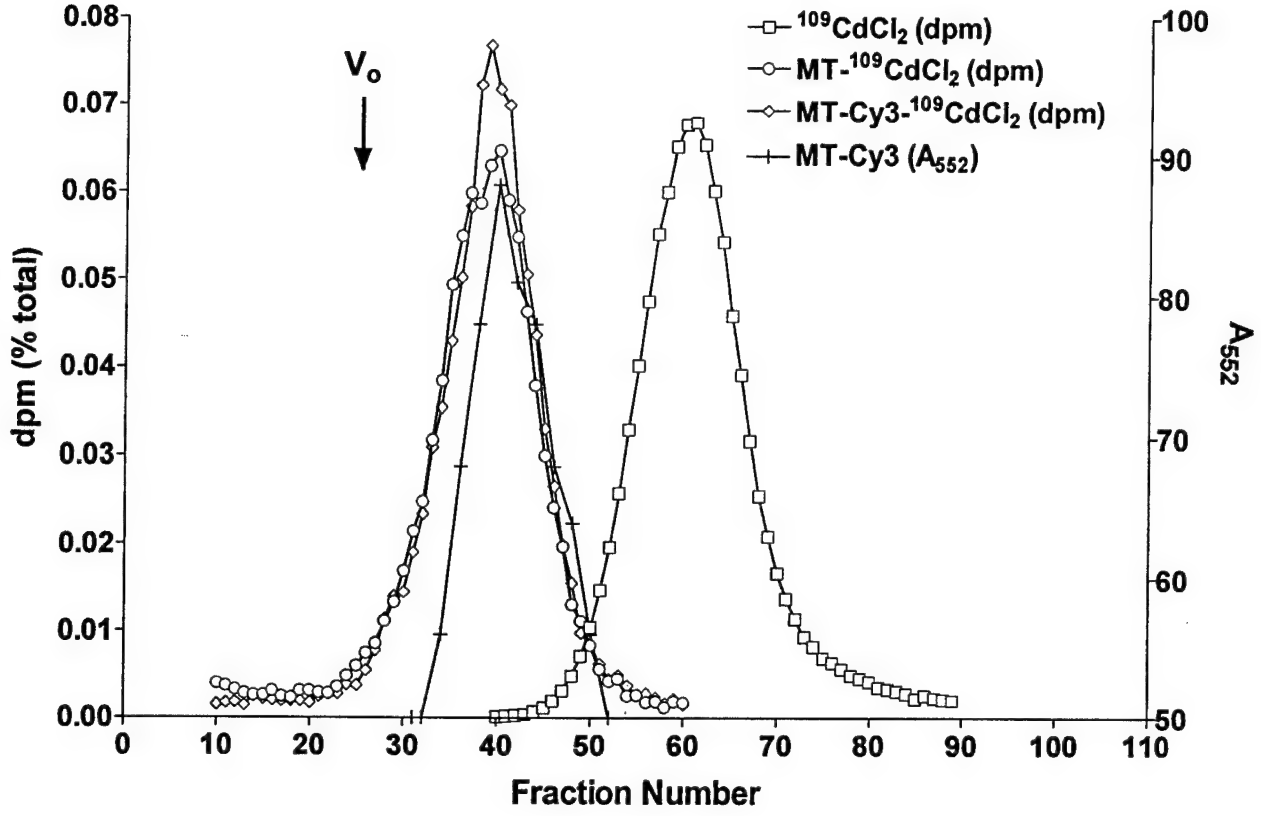

Figure 1. Woo et al.

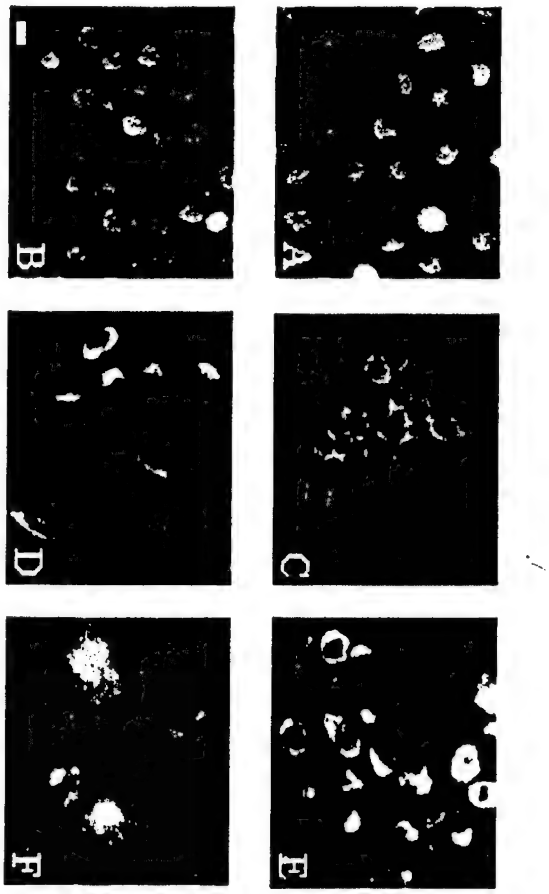

Figure 2. Woo et al.

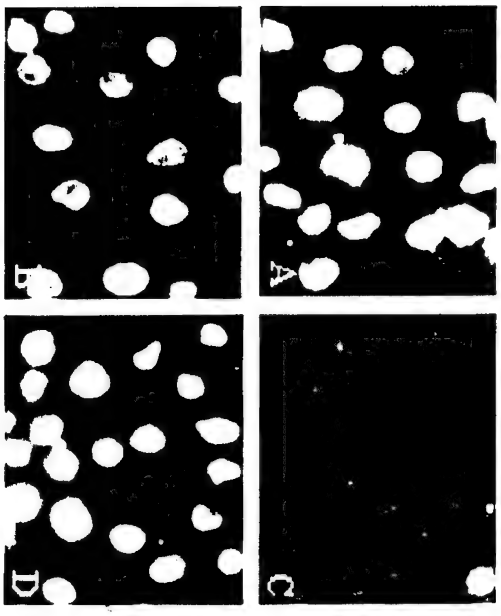

Figure 3. Woo et al.

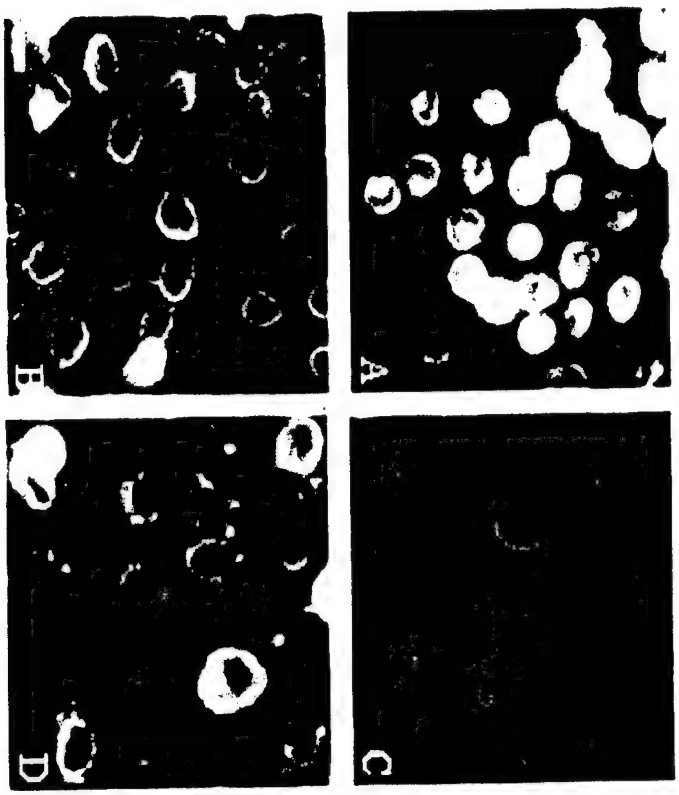

Figure 4. Woo et al.

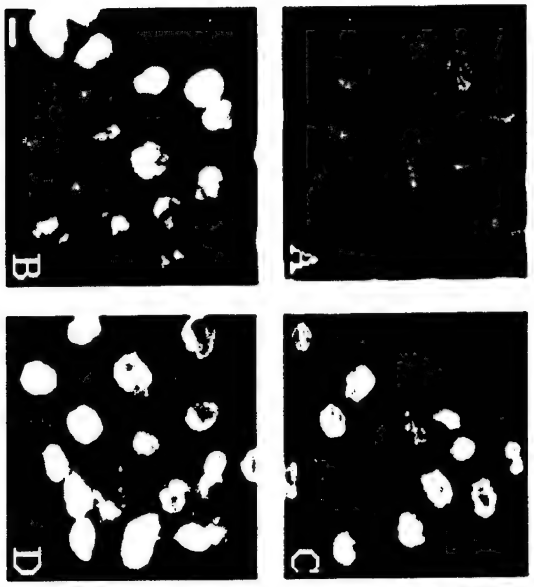

Figure 5. Woo et al.

Tamoxifen-Induced Dephosphorylation and Cleavage of the Retinoblastoma Protein During Apoptosis of Human Breast Carcinoma Cells.

Cheryl L. Fattman
Advisor: Q. P. Dou, Ph.D

Introduction

Control of cell number in a tissue or organism is determined by a balance between cell proliferation and cell death. Apoptosis, is a very specific process by which cells essentially commit suicide. Many factors can modulate induction of this process including growth factors, intracellular mediators of signal transduction, and nuclear proteins regulationg gene expression, DNA replication, and the cell cycle (1). Apoptotic cells undergo a stereotypical set of changes including cell shrinkage, condensation and fragmentation of the nucleus, and cleavage of nuclear DNA that make this a unique form of cell death (2). The mechanism by which these changes occur is still largely unknown but recent investigations have indicated that a family of proteases are essential components of the cell death pathway. These proteases, known as caspases, have been evolutionarily conserved from worm to man and have been shown to degrade proteins necessary to maintain the cell growth/cell death balance (3). However, disruption of this balance, through inappropriate apoptosis, can lead to such diseases as cancer (too little cell death) or AIDS (too much cell death).

Relevance to Breast Cancer

Recently, it has been found that many genes which regulate tumorigenesis are involved in the process of apoptosis. The p53 gene, now one of the most commonly detected abnormalities in human cancer, has been recognized as an important modulator of apoptosis (4). One of the functions of p53 may be to induce apoptosis even in the presence of oncogenic triggers. It has been found that loss of p53-dependent apoptosis correlates with tumor aggressiveness and that mutation of p53 is associated with poorer prognosis in many of the most common human malignancies, including breast tumors.

The p53 gene may control apoptosis by controlling the activity of a second protein, the retinoblastoma protein (RB). Mutations of RB were first identified through examination of hereditary forms of retinoblastoma. Since then, the mutant form of RB has been have been associated with breast carcinomas, lung carcinomas and prostate carcinomas, among others. RB is one of the major "checkpoint" proteins in the cell and its activity is regulated by phosphorylation. Under normal conditions, RB is in its active, unphosphorylated state (p110/RB), and prevents the cells from traversing the cell cycle. Conversely, inactivation of RB by phosphorylation (p120/RB), allows the cells to progress through the cell cycle (5). The p53 protein plays a role in determining the phosphorylation state of RB by regulating levels the inhibitors of the kinase enzymes (4). However, when

cells are induced to undergo apoptosis, the p120/RB is first dephosphorylated to a unique p115/RB. RB is subsequently cleaved into three fragments, p48/RB, p68/RB and p112/RB by a protease with properties of a caspase enzyme (6-8).

Research Performed

Our lab has previously investigated the role of RB in the process of anticancer drug-induced apoptosis (6-8). When human leukemic HL-60 or U937 cells were treated for 3h with a variety of anticancer agents, such as etoposide (VP-16) or cytosine arabinoside (Ara-C), RB was converted in the cells from the p120/RB to the p115/RB. In addition, the p48/RB, p68/RB and p112/RB cleavage fragments were detected a short time after RB dephosphorylation (5h). We believe these fragments are a result of proteolysis by a caspase enzyme. All of these processes were associated with the nuclear condensation and DNA fragmentation, common apoptotic indicators.

More recently, I have been investigating the role of RB in antiestrogen-induced apoptosis of two different breast cancer cell lines: MCF-7 and MDA-MB-231. These cell lines differ in both their estrogen receptor status (MCF-7 cells are ER+, while MDA-MB-231 cells are ER-) and their p53 levels (MCF-7 cells have wild-type p53, while MDA-MB-231 cells overexpress mutant ,non-functional p53). I have shown that these two cell lines are equally sensitive to 50mM tamoxifen since both lines show the characteristic morphological changes associated with apoptosis at ~ 4h of treatment. In addition, I have shown that dephosphorylation of RB correlated well with the early stages of apoptosis, while generation of the cleavage fragments occurred at a later time. I also observed that RB cleavage occurred more rapidly in the p53-negative MDA-MB-231 cells, occurring approximately 12h before cleavage in p53-positive MCF-7 cells. Tamoxifen treatment of either cell line, however, did not produce an increase in p53 protein levels nor a subsequent increase in the kinase inhibitor p21. These results indicate that activation of the caspase-mediated pathway of apoptosis and the prior event of RB dephosphorylation appears to be independent of p53 status.

Future Studies: Future studies will focus on mapping the cleavage site used by the caspase enzymes to generate the p48/RB and p68/RB fragments. Other studies will examine the functional role of RB cleavage as a regulator of apoptosis.

Presentation of Work:

1.) "Dephosphorylation of RB during breast cancer apoptosis". Invited Speaker, Magee Women's Hospital, June 1997.

2.) Fattman, C.L., An, B., Sussman, L., and Dou, Q.P. (1997) Tamoxifen-induced p53-independent dephosphorylation of the retinoblastoma protein during apoptosis of human breast carcinoma cell. Future presentation, 2nd annual Biomedical Graduate Student Symposia, November 1997.

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The Requirement for ATP and Specific Hsc70·DnaJ Homolog Interactions for Protein Translocation into the Yeast Endoplasmic Reticulum

Amie Jo McClellan
Advisor: Jeffrey Brodsky, Ph.D.

Introduction

In order to arrive at their ultimate destinations, integral membrane proteins, secreted polypeptides, and resident proteins of most intracellular organelles must first successfully traverse the secretory pathway. Included in this range of molecules are peptide hormones, growth factors and their receptors, and enzymes necessary for homeostasis. Thus, one can see why the proper localization of the proteins that utilize the secretory pathway is necessary for normal metabolic growth. In fact, the mislocalization of certain secreted proteins has been shown to be one cause of metabolic disorders such as cancer, cystic fibrosis, heart disease, and diabetes. The first committed step in the secretory pathway is the translocation of proteins into the ER.

Relevance to Breast Cancer

Proteins that traverse the secretory pathway play a necessary role in cancer growth and metastasis. For example, the invasive and metastatic abilities of tumor cells are promoted by the secretion of such molecules as collagenases, matrix-degrading proteases, and motility factors (1, 2). Also, the division of human breast cancer cells and glioblastoma cells is stimulated by the secreted growth factor IGF-1 (3, 4), and melanoma cell growth is activated by the secretion of interleukin-8 (5).

In a recent study, the non-tumorigenic EPH4 cell line, derived from spontaneously immortalized mouse mammary cells, was examined for alterations in response to thyroid hormone (T_3; 3, 5, 3' - L - triiodothyronine) (2). Investigators uncovered several actions of T_3 on mammary epithelial cell function that are likely to correspond to the development of breast cancer, including metastatic potential. The effects of T_3 included increases in the secretion of the extracellular matrix proteases stromelysin 1 and stromelysin 2 (ST-1 and ST-2), enhancement of the collagenolytic activity of the cells, and loss of cell polarity as noted by the mislocalization of several apical and basolateral proteins. All of these effects of T_3 are dependent upon the level of expression and proper secretion of the T_3 receptor, TRα-1. As protein translocation into the ER is the first committed step in protein secretion, elucidating the mechanism of protein translocation is a necessary goal precluding the design of chemotherapeutic agents to selectively inhibit the secretion of cancer promoting factors, such as TRα-1, ST-1, ST-2, and T_3. The research proposed in this application will provide a means to this end.

Another recent study examined the link between GRP78/BiP suppression and the inhibition of tumor progression (6). In B/C10ME fibrosarcoma cells, the stess response induction of GRP78/BiP correlates with resistance to cell-mediated lysis by cytotoxic T lymphocytes. Also, *in vivo* tumor progression is greatly inhibited in a B/C10ME clonal cell line stably transfected with amplified copies of a *grp78/BiP* antisense vector. Based upon these results, GRP78/BiP may play a role in the control of tumor cell apoptosis. The more that is known about the function of BiP in the cell, the greater the chance that we can one day alter its regulation and overcome the stress response induction of BiP that promotes tumor progression.

Research Performed

In addition to the integral membrane and membrane-associated proteins that comprise the translocation machinery at the ER membrane, translocation requires the function of cytosolic and ER lumenal heat shock cognate proteins (hsc70s). Hsc70s couple ATP binding and release to repeated cycles of protein binding and release. Thus, hsc70s probably interact with and maintain translocating proteins in an unfolded and, therefore, translocation-competent state as they traverse the ER lipid bilayer. In addition, these hsc70s interact with members of the DnaJ family of molecular chaperones that are also required for translocation. Although various models have been suggested to explain how these chaperones facilitate protein translocation, their mode of action at the molecular level remains obscure. The goal of my project has been to define the role(s) of these hsc70 and DnaJ molecular chaperones during protein translocation into the ER.

To this end, I have purified the cytosolic hsc70, Ssa1p, the ER lumenal hsc70, BiP, the cytosolic DnaJ homolog, Ydj1p, and a GST-fusion protein containing the ER-lumenal DnaJ-like domain of the ER integral membrane protein Sec63p (GST-63Jp). I have found that while Ssa1p and BiP exhibit identical rates of ATP hydrolysis in a steady-state kinetic analysis, the ATPase activity of Ssa1p is stimulated by Ydj1p ~12-fold, but Ydj1p stimulated the ATPase activity of BiP only 2-fold. Additionally, in a native gradient PAGE assay, Ydj1p and ATP elicited the release of a radiolabeled unfolded protein substrate, ^{125}I-CMLA, from Ssa1p, but not from BiP. The specificity of DnaK homolog·DnaJ homolog interactions was further established by showing that BiP, but not Ssa1p, binds to GST-63Jp in an ATP-dependent manner. Therefore, the specificity of these hsc70s for their compartment-specific DnaJ homologs is the basis of their topologically-restricted functions in protein translocation.

To identify essential functions of the lumenal hsc70, BiP, four dominant lethal mutants of BiP have also been purified. All four mutant proteins are the result of single amino acid substitutions in the ATPase domain of BiP. Because it is known that BiP forms an ATP-dependent complex with the translocation machinery via the DnaJ-domain of Sec63p, BiP and ATP hydrolysis may regulate protein translocation. I have discovered that the dominant lethal mutants of BiP are essentially devoid of ATPase activity in comparison to wild type BiP, and they are also impaired in their ability to bind ATP. The mutant BiP proteins are also unable to associate with GST-63Jp in an ATP-

dependent manner. The ability of the BiP mutants to bind to an unfolded protein substrate, ^{125}I-CMLA , is not compromised. However, the mutant proteins may be defective for release of protein substrate, but an assay demonstrating the release of unfolded protein substrates from wild type BiP has not yet been established. Most interestingly, all four dominant lethal BiP mutants inhibit the translocation of the yeast mating pheromone precursor, ppαf, in *in vitro* translocation assays. This inhibition of translocation is observed using both equimolar amounts of wild type and mutant protein, and when wild type protein is present in great excess. As wild type and all four mutant proteins exist as monomers and dimers when assessed by native PAGE, perhaps nonfunctional wild type·mutant dimers are formed *in vivo* and this results in the lethal phenotype.

In addition, I have collaborated with the laboratory of James Pipas and demonstrated that the J-domain of SV40 T antigen is able to functionally interact with Ssa1p. That is, the T antigen J-domain stimulates the ATPase activity of Ssa1p, and, in combination with ATP and Mg^{2+}, T antigen effected the release of Ssa1p from ^{125}I-CMLA. SV40 T antigen has been shown to transform mammalian cell lines, and is known to interfere with the normal cellular function of members of the retinoblastoma family of proteins and the tumor suppressor p53. The system I have established can differentiate between functional and non-functional T antigen J domains, and may lead to new discoveries regarding the mode of action of SV40 T antigen in tumorigenesis.

These and future experiments will serve to elucidate the role(s) of hsc70s in the translocation of proteins across the ER membrane. This information will further clarify the mechanism of protein secretion and may aid in the development of new therapies and diagnoses for diseases that result from the mislocalization of proteins that utilize the secretory pathway.

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Functional Interactions Between the Retinoblastoma Protein and Components of the Transcription Initiation Complex

Jennifer L. Siegert
Advisor: Paul D. Robbins, Ph.D.

Our lab has recently demonstrated that the retinoblastoma tumor suppressor protein, Rb, is able to bind directly to TAF||250, the largest subunit of the TFIID basal transcription factor. TAF||250 was originally identified as the cell cycle-regulatory protein, CCG1, and its mutation has been shown to result in cell cycle arrest in the G1 phase. We have mapped the domains in Rb important for its association with TAF||250 to two independent regions, both of which fall within regions of Rb that have been shown to be mutated in several human tumors, including breast. In addition, I have mapped the regions in TAF||250 which are important for binding to Rb, and the results suggest several potential functions for the interaction of Rb with TAF||250. The large pocket of Rb binds strongly within a recently-characterized kinase domain in TAF||250, and both the large pocket and amino terminus of Rb bind within or near to another recently-characterized catalytic domain, for histone acetyltransferase activity.

The intrinsic kinase activity of TAF||250 is capable of autophosphorylation and has also been shown to be important for the specific transphosphorylation of the RAP74 subunit of the basal Pol II transcription factor, TFIIF. I have performed a series of in vitro kinase assays with purified recombinant proteins and have demonstrated that Rb, specifically the large pocket, is able to inhibit both the autophosphorylation of TAF||250 as well as the phosphorylation of RAP74. In addition, two different tumor-associated mutations within Rb's large pocket, a Cys-to-Phe point mutation at amino acid 706 and deletion of exon 22, rendered Rb functionally dead for TAF||250 kinase inhibition, even though neither mutation had much of an affect on Rb's ability to bind to TAF||250, particularly within the kinase domain. The inhibition of TAF||250 kinase activity by Rb could potentially represent a mechanism of transcriptional repression, in that the phosphorylation of RAP74 by TAF||250 is believed to possibly either affect the recruitment of RNA polymerase to the promoter or modulate its elongation activity. In addition, although TAF||250 is apparently not required for general transcription in vivo, it does appear to have a specialized role in transcriptional regulation of cell cycle progression and growth control, similar to the role of Rb. Interestingly, the yeast homolog of TAF||250 was very recently shown to be required for transcription of G1/S cyclin genes, which are responsible for regulating the phosphorylation state, and thus the activity, of Rb. In the future, I plan to continue to characterize the functional interaction between Rb and TAF||250, with the eventual hope of elucidating the mechanism responsible for Rb's ability to inhibit the kinase activity of TAF||250, as well as determining the effect that this inhibition by Rb might have on transcription. Furthermore, I will attempt to further define the defect associated with the naturally-occurring Rb mutants described above, and how this may be contributing to tumorigenesis.

The histone acetyltransferase (HAT) activity of TAF||250 is thought to potentially play an important role in controlling the transcription machinery's access to nucleosome-bound promoter sequences. I have made some preliminary attempts to determine whether Rb is able to affect TAF||250's HAT activity, but do not yet have any definitive results. In the future, I will be trying several different experimental approaches which I hope will address this question satisfactorily. Histone acetylation is currently a

very exciting topic in the field of transcription, and if Rb could be linked to modulation of HAT activity, this finding might explain a lot of Rb's regulatory capabilities related to transcription.

Finally, using several different in vitro experimental assays, I have also seen specific binding of Rb to another component of the Pol II basal transcription complex, TFIIH. In experiments thus far, I have seen an apparent effect of Rb on the helicase activity of TFIIH, an activity which is associated with promoter clearance during the transition from transcription initiation to elongation and also appears to play a role in DNA excision repair. However, the results have been somewhat inconsistent between preparations of TFIIH of variable purity. Thus, I will be attempting to determine what might be the explanation for the inconsistencies, and at the same time continuing to pursue the question of whether Rb is able to affect any aspect of transcription initiation directly related to TFIIH function. For the latter, we will be collaborating with another lab which is readily equipped to carry out in vitro transcription assays, and we will supply them with purified Rb proteins to test with TFIIH. A functional interaction between Rb and TFIIH, which plays many important parts in transcription, could provide an additional mechanism of transcriptional regulation by Rb, and thus another means for Rb to control cell cycle progression and function as a tumor suppressor.

Publications:

Siegert, J.L. and Robbins, P.D. (submitted) Rb inhibits the intrinsic kinase activity of the TBP-associated factor, $TAF_{II}250$.

Shao, Z., Siegert, J.L., Ruppert, S. and Robbins, P.D. (1997) Rb interacts with $TAF_{II}250$/TFIID through multiple domains. Oncogene **15**:385-392.

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Invited Oral Presentations:

The retinoblastoma protein inhibits the intrinsic kinase activity of the TBP-associated factor, $TAF_{II}250$. *Cancer Genetics & Tumor Suppressor Genes*, FACSI Conference, Frederick, MD (1997).

Rb inhibits the intrinsic kinase activity of the TBP-associated factor, $TAF_{II}250$. Department of Molecular Genetics & Biochemistry Retreat, University of Pittsburgh School of Medicine, Pittsburgh, PA (1997).

Abstracts:

Siegert, J.L. and Robbins, P.D. (1997) The retinoblastoma protein inhibits the intrinsic kinase activity of the TBP-associated factor, $TAF_{II}250$. Poster presented at *Mechanisms of Eukaryotic Transcription*, Cold Spring Harbor Laboratory, NY.

Siegert, J.L., Shao, Z., Ruppert, S., and Robbins, P.D. (1996) Targets for the retinoblastoma tumor suppressor within the basal transcription initiation complex." Poster presented at *The Cell Cycle*, Keystone Symposia on Molecular and Cellular Biology, Taos, NM.

Enhancement of T-Lymphocyte Effector Function by Constitutive Expression of Protein Kinase C

James T. Snyder
Advisor: Olivera Finn, Ph.D.

Most tumor antigens defined to date fall into a group known as tissue differentiation antigens. What that means is that they are normally expressed only transiently during a particular stage of differentiation and not in normal fully differentiated tissues. Malignant transformation of these tissues appears to lead to reexpression of these proteins, which can then be used as tumor specific antigens. The strength of these antigens and their ultimate utility in tumor immunotherapy depends on how they were originally perceived by the immune system and if tolerance to them developed. In my project we are testing a general hypothesis that autoimmunity to well defined antigens (such as MUC1, p53 or Her2/neu in breast cancer) can be used as effective tumor immunity. To test this general hypothesis we chose a test system and formulated a hypothesis specific to it:

Hypothesis: Disruption, via disregulated expression of protein kinase C (PKC), of the normal signaling processes that lead to elimination of autoreactive T cells, may result in improved immune surveillance of tumors: We have developed lines of transgenic mice in which PKC is expected to be constitutively expressed in T cells at different stages of development. In one line, PKC-betaII is expressed from the lck proximal promoter, which should result in constitutive expression of PKC in immature T cells developing in the thymus. In another line of transgenic mice, PKC-betaII is expressed from the lck distal promoter, which should result in constitutive expression of PKC in mature, peripheral T cells. It was our expectation that the altered expression of PKC in these mice will lead to the disruption of the normal signaling processes in T cells, and this may in turn result in failure to eliminate potentially autoreactive T cells in these mice. This will give us a model to determine whether tumor immunity is equivalent to autoimmunity.

Progress on PKC transgenic mice: Mice from both the strain in which PKC is expressed from the lck distal promoter and the strain in which PKC is expressed from the lck proximal promoter have exhibited phenotypic changes. In mice in which PKC is expressed from the lck proximal promoter, which should produce overexpression of PKC in the early stages (prior to CD3 expression) of T cell development in the thymus, there appear to be gene dose and age dependent changes in gross phenotype as well as in the lymphocyte repertoire. Homozygous mice from this strain, as well as some of the older founder mice, develop splenomegaly, and, in some cases, enlarged mesenteric nodes. There is also evidence of infiltration of the liver and kidneys with lymphocytes in some of these mice. Thymic T cell populations in these mice are also unusual. Generally, we see a shift away from the CD4 CD8 double positive population towards either a double negative population or towards the CD4 single positive

population. There are reduced numbers of CD3 positive cells in the spleens of these mice, in spite of the fact that their spleens are enlarged. In general, these changes have not been observed in the F1 and F2 heterozygous mice from this strain, indicating that this may be a gene dose related effect. We are currently in the process of determining whether these mice retain certain T cell receptors (V beta) which are normally deleted in these mice due to potential autoimmune effects. If so, these mice will, as we expected, be a good model of tumor immunity as it relates to autoimmunity.

In mice in which PKC is expressed from the lck distal promoter, which should produce high PKC expression in mature, peripheral T cells, we have also observed some age-related phenotypic changes. All of the founders from this strain exhibited changes in lymphoid organs. in most cases, the lymph nodes were greatly enlarged. One founder also exhibited splenomegaly, as well as an enlarged thymus. Two other founders, however, had very small spleens. Splenocyte populations were normal in two of the founders, but one founder (which had a small spleen) had reduced numbers of CD3 positive cells , while another founder (which had an enlarged spleen) had increased numbers of CD3 positive cells. Two of the founders had reduced numbers of CD4 CD8 double positive cells in the thymus (only 8-20%), and increased numbers (50-80%) of double negative cells. At least one founder exhibited infiltration of the liver with mononuclear cells. These changes have not been observed in heterozygous F1 and F2 mice from this strain, but we have observed similar effects in the homozygous mice from this strain. We have evidence that these mice have increased T cell function compared to normal controls (i.e., they show increased T cell responsiveness to mitogenic stimuli). This, combined with the evidence for autoimmune effects, may make these a good model of a "hyperimmune" mouse. We will determine if these mice have increased responsiveness to tumor antigens, as well.

Breast cancer model: In the coming year,we are planning to breed mice from both these strains with Neu or p53 transgenic mice, which spontaneously develop breast tumors. We can then determine whether the double transgenic mice have improved immune surveillance of tumors, and whether this tumor immunity is correlated with autoimmune effects in these mice.

Abstracts Presented:

The Role of Protein Kinase C in T-Lymphocyte Activation and Development. J.T. Snyder, II and O.J. Finn, Department of Molecular Genetics and biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA. Presented at the Keystone Symposium on Lymphocyte Activation, Hilton Head, SC, March 20-26, 1996.

The Role of Protein Kinase C-Beta in T Lymphocyte Activation and Tolerance Induction. J. T. Snyder II and O.J. Finn. Presented at the AAAAI/AAI/CIS Joint Meeting, February 21-26, 1997.

APPENDIX

Second Annual Pittsburgh Minisymposium

on Basic and Translational Research in Breast Cancer

Saturday, August 16, 1997

Center for Environmental and Occupational Health and Toxicology
University of Pittsburgh

Sponsored by
the Comprehensive Breast Cancer Program

a Joint Program of
the University of Pittsburgh Cancer Institute
and
Magee-Womens Hospital

Program

Continental Breakfast

8:30-8:45 *Poster Hanging*

8:45-8:50 **Stephen Grant:** *Welcoming Remarks*

8:50-9:00 **Victor Vogel:** *Introductory Remarks*

Platform Session 1: Cell Biology and Molecular Etiology of Breast Cancer

9:00-9:15 **Amal Kanbour-Shakir:** *Update on the In vivo Behavior of Breast Tumor Cells*

9:15-9:30 **Qing-Ping Dou:** *Update on Apoptosis Regulation in Breast Cancer*

9:30-9:45 **Alakananda Basu:** *Update on TNF-α Signaling in a Breast Cancer Model*

Platform Session 2: Molecular Epidemiology and Biomarkers

9:45-10:05 ***Joel Weissfeld:*** *Evaluation of Screening Mammography*

10:05-10:25 ***Lewis Kuller:*** *Hormones and the Epidemiology of Breast Cancer*

10:25-10:45 ***Marjorie Romkes:*** *Drug Metabolizing Enzymes as Risk Factors in Breast Cancer*

10:45-11:05 ***Adedayo Adedoyin:*** *Estrogen Metabolism—Role and Modification during Breast Carcinogenesis*

11:05-11:25 ***Wendy Rubinstein:*** *BRCA Mutations, Prevention Decisions and Mental Health*

Poster Session

11:30-12:00

Lunch

12:00-1:00 (by Thornton and Thornton: The Consummate Kitchen)

Featured Speaker

1:00-2:00 ***Daniel Stoler:*** *Chromosomal Genomic Instability in Cancer*

Platform Session 3: Genetic Instability in Breast Cancer

2:00-2:10 ***Jean Latimer:*** *Update on Loss of DNA Repair in Breast Carcinogenesis and Tumor Progression*

2:10-2:20 ***Stephen Grant:*** *Update on Somatic Mutation and Breast Carcinogenesis*

Coffee Break

2:20-2:40

Platform Session 4: New Paradigms in the Treatment of Breast Cancer

2:40-3:00 ***Herb Jacob:*** *New Paradigms in Breast Cancer Therapy*

3:00-3:10 ***Billy Day:*** *Update on New Drugs: Tubulin Interactive Agents*

3:10-3:20 ***Ken Giuliano:*** *Update on High-Throughput In Vitro Assays for Screening of Anticancer Agents*

3:20-3:30 ***Elizabeth Hiltbold:*** *Update on Clinical Trials with a Mucin Peptide Vaccine*

3:30-3:50 ***Kristina Gerszten:*** *Radiobiology and Radiosensitivity*

Concluding Remarks

3:50-3:55 ***Stephen Grant***

3:55-4:00 ***Victor Vogel***

CEOHT Tours

William Bigbee, Billy Day, Stephen Grant, Phouthone Keohavong, Marjorie Romkes

Second Annual Pittsburgh Minisymposium

on Basic and Translational Research in Breast Cancer

Poster Session

***James T. Snyder**, and **Olivera J. Finn** Department of Molecular Genetics and Biochemistry, University of Pittsburgh*

The Role of Protein Kinase C in T-Lymphocyte Activation and Development

***Barbara J. Henry, Meu Liu, Orest T. Macina, Gilles Klopman, Herbert S. Rosenkranz**, and **Stephen G. Grant** Departments of Environmental and Occupational Health and Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh*

Short-Term Assays of Somatic Segregation: Association with the Molecular Etiology of Cancer

***Tavio G. Ijaduola, Sandra Blakowski, Arnold Meisler, Stephen Winters**, and **Wendy S. Rubinstein** Departments of Medicine, Obstetrics, Gynecology and Reproductive Sciences, Human Genetics, and Endocrinology, University of Pittsburgh, and the Veterans Affairs Medical Center, Pittsburgh*

Genetics of Male Breast Cancer—A Population-Based Study

***Barbara J. Henry, Gilberto Fregoso, William L. Bigbee, Robert Branch**, and **Marjorie Romkes** Department of Environmental and Occupational Health and the Center for Clinical Pharmacology, University of Pittsburgh*

Impact of Genetic Polymorphisms in Xenobiotic Metabolizing Enzymes on Inter-Individual Variations in the Levels of In Vivo Genetic Damage in Humans

1997 Attendees

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the role of specific estrogen metabolizing enzymes in breast cancer pathogenesis and how their activities may be modulated to influence the disease

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apoptosis of MCF-7 and MDA-MB231 cells

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apoptosis in epithelial cells

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tumor necrosis factor-α signaling in breast cancer

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telomerase expression in early breast lesions (DCIS); vitamin D receptor polymorphisms and breast cancer risk; chemoprevention

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clinical trials of adjuvant therapy for early stage breast cancer; novel therapies for advanced disease; serial analysis of gene expression in breast cancer

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computational design and discovery, synthesis, cell culture evaluation and metabolism of potential anti-breast cancer agents, especially tubulin- interactive agents; mechanisms of hormone-induced breast carcinogenesis

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molecular mechanisms of programmed cell death (apoptosis) and drug resistance

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mutagenesis and carcinogenesis, apoptosis, biomonitoring of genotoxic therapy, radiosensitivity, genetic predisposition to cancer

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breast cancer vaccines based on the mucin molecule

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sequencing of chemotherapy and radiotherapy; tumor radiosensitivity after neoadjuvant therapy

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live-cell-based drug discovery tools

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T-helper cell response to MUC-1 mucin

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genotyping and mRNA expression of biotransformation enzymes

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DNA repair and breast cancer; primary culture of normal and tumor-derived epithelial cells

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genetics of male breast cancer

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new paradigms in breast cancer therapy: angiogenesis inhibition, basement membrane destruction, mutation, mechanisms of drug resistance

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biology and histopathology of breast disease and cancer

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p53 gene mutations as biomarkers for breast cancer

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the role of dendritic cells in the tumor immunogenicity of breast cancer

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evaluation of the role of phase I/II enzymes in breast cancer risk

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genetic epidemiology of breast cancer; early molecular detection of breast cancer using nipple aspirate fluid; medical and psychological outcomes following genetic testing; genetic counseling and education; genetic determinants of male breast cancer; risk assessment

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immortalization of T cells specific for breast cancer, analysis of mouse models of immunology

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evaluation of screening mammography

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chemoprevention of breast cancer; effect of differentiating agents on early breast lesions

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development of in vivo models (especially orthotopic and metastatic) for human breast cancer, and employment of such models for the study of basic biology and preclinical applications

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interleukin-6 as a tumor marker in patients with breast cancer

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variation in steroid hormone metabolizing enzymes and risk of breast cancer in post-menopausal women

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Free radical reactions in biology and medicine, genotoxicity of free radicals

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Breast Cancer Case Conference Seminar Schedule	Thursdays, 4-5 PM	Clinic Conference Room, 0 Level Magee Womens Hospital
Speaker	**Date**	**Title**
Kenneth Korzekwa, Ph.D. University of Pittsburgh Recipient, UPCI Breast Cancer Pilot Project Grant	5/15/97	Steroid Metabolism by the Cytochrome P450 Enzymes.
Gini Fleming, M.D. Medical oncologist, University of Chicago (recruitment candidate)	5/22/97	Chemotherapy for Metastatic Breast Cancer.
Nancy Davidson, M.D. Professor of Medicine Johns Hopkins Oncology Center	5/29/97	High dose therapy and breast cancer.
Marjorie Romkes, Ph.D. University of Pittsburgh Recipient, UPCI Breast Cancer Pilot Project Grant	6/5/97	Cytochrome P450 mRNA expression in human breast tissue.
Quin-Ping Dou, Ph.D. University of Pittsburgh Recipient, UPCI Breast Cancer Pilot Project Grant	6/19/97	Apoptosis regulation and breast cancer.
Jean Latimer, Ph.D. University of Pittsburgh, MWRI Recipient, UPCI Breast Cancer Pilot Project Grant	7/17/97	In vitro behavior studies of normal mammary epithelial cells and breast tumor cells; what can these studies tell us about in vivo behavior?
Adam Brufsky, M.D., Ph.D. Medical oncologist University of Pittsburgh	8/21/97	Serial analysis of gene expression: Potential applications for breast cancer

Speaker	Date	Title
Stephen Grant, Ph.D. University of Pittsburgh Recipient, UPCI Breast Cancer Pilot Project Grant	9/4/97	Differential patterns of apoptosis in breast cancer cells.
Clinical Journal Club	9/18/97	Recent NSABP Data
Clare Faul, M.D. Magee Womens Hospital Radiation oncologist	10/2/97	Timing of radiation and chemotherapy in both early and advanced breast cancer.
Clinical Journal Club	10/23/97	Radiation therapy update (adjuvant postoperative chest wall irradiation)
Lynne Bemis, Ph.D. AMC Cancer Research Center Denver, Colorado	10/30/97	Lipocalin and MMP-9 based mechanisms of metastasis.
Amal Kanbour-Shakir, M.D., Ph.D. Magee Womens Hospital Pathologist	11/6/97	Selected cases of the breast care consultation center.
Clinical Journal Club	11/20/97	Medical oncology update
Jill Siegfried, Ph.D. University of Pittsburgh Recipient, UPCI Breast Cancer Pilot Project Grant	12/4/97	Hepatocyte growth factor and breast cancer.
Clinical Journal Club	12/18/97	Surgical update
Clinical Journal Club	1/22/98	Radiation therapy update
Phouthone Keohavong, Ph.D. University of Pittsburgh Recipient, UPCI Cancer Award	2/5/98	Ras mutations in colorectal cancers; an important parallel with breast cancer.
Jim Roberts, M.D. Director, Magee Womens Research Institute	2/19/98	The epidemiological connection between preeclampsia and breast cancer.
Kathy Purcell, M.S.W., L.S.W. Oncology Social Worker Magee Womens Hospital	3/5/98	Psychosocial issues for patients dealing with breast cancer.
Lisa Begg, Dr.P.H. Director of Epidemiology Allegheny General Hospital	4/2/98	Breast cancer and nutrition; possible connections to chemoprevention